Spectroscopic analysis and cytotoxic activity of quas-sinoid isolated from the seeds of *Brucea javanica* on Hela cell

Spektroskopik dan aktivitas sitotoksik kuasinoid hasil isolasi dari biji buah *Brucea javanica* terhadap sel HeLa.

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Abstract

The quassinoids from *Brucea javanica* exhibit biological activities as antimalarial, antitumor, antiviral and antiamoebic activities. These quassinoids having a promising antitumor activity.This research was aimed to isolated quassinoid (bruceine A, MW 522 g/mol) compound from *B. javanica* and to investigate the cytotoxicity of bruceine A under *invitro* conditions in Hela cells. The cytotoxicity assays using MTT assay. The bruceine showed a good cytotoxic activity in *Hela* cells at 24 hours with doxorubicine as a positive control. Structural elucidation of bruceine is done by using UV and FT-IR spectra, NMR spectra recorded on Tesla av600, (400 MHz) ^{1}HNMR in pyridine (D_5), some in Gyro (300 MHz) ^{1}HNMR in acetone-(d6), DMSO-(d6) and $CdCl_3$, ^{13}CNMR, 75 MHz, mass spectra on biospectrometry LC-MS. Analytical HPLC retention time,t_R was 4.617 minutes (Met/$H_2$0) 60:40. Its Molecular formula was determined to be (C_{26} H_{34} O_{11}) by the $[M+H]^+$ ion peak at m/z 523 and m/z 1045 confirmed by High Resolution mass spectra Orbitrap (HRESIMS). From the results demonstrated that could be bruceine A compound refers to our spectral data. Its has activity in IC_{50} value 191,5 µM on Hela Cells under *invitro* conditions.
Key words: Quassinoid, *B. javanica,* Brusein A, Spectroscopic, Anticancer

Abstrak

Kuasinoid yang terdapat dalam *Brucea javanica* telah diketahui memiliki aktivitas biologik sebagai antimalaria, antitumor, antivirus serta antiamuba. Kuasinoid terutama karena aktivitasnya sebagai antitumor telah menjadi perhatian dan sangat menjanjikan. Penelitian ini bertujuan untuk mengisolasi kuasinoid (brusein A, MR 522 g/mol) dari *B. javanica* serta mengkaji aktivitas sitotoksis brusein A terhadap sel HeLa secara invitro. Uji sitotoksik menggunakan metode MTT test. Brusein A menunjukkan aktivitas sitotoksik yang baik terhadap sel HeLa pada perlakuan 24 jam menggunakan bahan obat doksorubisin sebagai kontrol positif. Elusidasi struktur brusein A telah dilakukan menggunakan UV; FT-IR, LC-MS, HPLC dan spekctra NMR dengan Tesla av600, (400 MHz). Spektra ^{1}HNMR dilakukan dengan pelarut piridin (D_5), beberapa hal dengan Gyro (300 MHz) dalam aseton -(d6), DMSO-(d6) dan $CdCl_3$, Spektra karbonnya dengan ^{13}CNMR (75 MHz). Spektrum massa menggunakan biospectrometry LC-MS. Analisis HPLC menunjukkan waktu retensi brusein *(retention time)*,t_R adalah 4.617 menit (Met/$H_2$0) 60:40. Formula Molekulnya yang diperoleh adalah (C_{26} H_{34} O_{11}) berasal dari limpahan ion $[M+H]^+$ pada m/z 523 dan m/z 1045 yang dikonfirmasikan dari spektrum massa resolusi tinggi *(High Resolution mass spectra)* Orbitrap (HRESIMS). Hasil

penelitian sesuai data spectra menunjukkan senyawa tersebut adalah bruceine A . Pada uji invitro aktivitas sitotosiknya (IC_{50}) pada sel HeLa adalah 191,5 µM.
Kata kunci: Kuasinoid ; *B. javanica,* Brusein A, Spektroskopik, Antitumor

Introduction

Brucea javanica locally are known as (buah makassar, kualot), has been used in "Jamu" traditional folk medicine in Indonesia to treat malaria, dysentery, and cancer (Syamsuhidayat, 1997). The seeds are known to be a rich of source quassinoids, the principle of bitter (Bray, 1987; Kim *et al*., 2004). *Brucea javanica* (L.) Merr belonging to the family of Simaroubaceae is a shrub which is distributed in Southeast Asia including Indonesia (Syamsuhidayat, 1997). The bioactive component of quassinoids from *Brucea javanica* have an interesting biological effect (O' Neill *et al*., 1985;1986 ; Wright *et al*., 1988; Su *et al*., 2002). There were javanicosides, flazin, bruceocides bruceantine, bruceine, brusatol and terpenoid blumenol A. Some with glycone or aglycone derivates. Yadanziosides was quassinoid glucosides. The mode of action based on inhibition of protein synthesis (Su *et al*., 2002; Cuendet, 2004). These quassinoids were reported for their potential to induce human promyelocytic leukemia (HL-60) cell differentiation and to inhibit cyclooxygenase-(COX-1), -2(COX-2) and 7,12 dimethyl benz[a]anthracence (DMBA)-induced lesions in a mouse mammary organ culture model. Bruceantinols and bruceines have been shown to possess as antibabesial activity with IC_{50} at 5 ng/mL (Subeki *et al*., 2007). Bruceantine and brusatol have antileukemic effects *induced* down-regulate *c-myc* protein levels (Greenwood *et al*., 2002). Some quassinoid have also been found to exhibit antiplasmodial activity against *Plasmodium falciparum* (O'Neill *et al*., 1985, Anderson *et al*., 1991).

Bruseantine, brusatol as well as brusein A have closely related in its structure. All possess a diosphenol (3-hydroxy- 3-en-2-one) group in the ring A (figure 1). The hydroxyl group in ring A attached to double bond of carbon and hydroxyls group in ring C. They have C-21 alkoxy chain but only ester side chain at C-15 make them different (Hitotsuyanagi, *et al*., 2004; Harigaya, *et al*., 1989). Therefore chemical reactivity of the ring A or C moiety can be observations. It can be changed with acyl groups or protecting groups. In spite of their promising anticancer, studies activity relationship (SAR) saw quite late progress. Furthemore study of isolated bruceine from *B. javanica* of Indonesia plant has not been published yet. Hence we were interested in to isolation of quassinoid that present in *B. javanica* and the possibility of Its semi-synthesis with pharmacophore mostly in acyl groups to have more anticancer activity.

In this study was planed to isolated quassinoid brucein A, Its structural elucidation and cytotoxic-anticancer activity on Hela Cells under *invitro* conditions.

Methodology

Plant Material

The seeds of *B. javanica* fruits were purchased and collected from Merapi Farma Herbal & Co., Yogyakarta, Indonesia. This sample was identified by Department of Biology, Faculty of Pharmacy UGM. Voucher of specimen no. FA/BF/146/ Ident./IX/09 was deposit at Department of Biology, Faculty of Pharmacy UGM. The seeds were dried and powdered before extraction.

Extraction and Isolation

The powdered seeds (5 kg) of *B. javanica* was defatted by hexane (5 L) for 3 days at room temperature. After filtering the hexane, the seeds were extracted using methanol (10 L) twice (3 days). The methanol extract then was filtered, then concentrated in a rotary evaporator to give a syrup, to which the equal volume of water was added. The aqueous solution was completely defatted with hexane 3 times and extracted again with dichlorometane 4 times. The organic layer was concentrated to give a residual, which was dissolved in methanol kept at 60 ^{o}C. (Sakaki *et al*., 1986). The methanolic solution was allowed to stand at room temperature to afford brusatol analog as a crystals, which was filtered off. The obtained precipitated can be removed and dried under vacuum to give amorf crystal after completely dried (ca 0,001% yield). Crystal can be separated by the Thin Layer Chromatography (TLC) to give brusatol analog or bruceine using elution ethyl acetate and chloroform (5:3).

Furthermore the filtrate above was evaporated to give an oily residue. Component of oily residue was separated by the following

procedure. Column A, 10 g of oily residue was subjected to separation by silica gel (100 g) column chromatography. The residue was eluted with 250 mL of Toluene-AcOEt (2:1) to give fraction 1, Toluene-AcOEt (1:1) to give fraction 2, AcOEt to give fraction 3. Column B, from the fraction 2 of Column A elution with PhH-AcOEt (1:1) gave fraction 1-3. The fraction 2 of Column B yielded brusatol analog as crystals on standing, which was filtered off (Sakaki *et al*., 1985).

Analytical and Structural Elucidation

Melting point was determined by using a scientific melting point apparatus Electrothermal 9100 array in open capillaries. UV spectra were recorded on UV Milton Roy spectrophotometer 3000, IR spectra were recorded on a Prestige 21 Shimadzu FTIR Spectrophotometer. NMR spectra were recorded on a Bruker 300 MHz and JNM ECA 500 MHz NMR in pyridine (d_5), acetone (d6), DMSO (d6) and mass spectral was obtained on biospectrometry Hitachi L 6200 LC-MS in methanol-water (80:20). Analytical HPLC Shimadzu 10AVP equipped with detector SPD-10AVP UV-Vis using Met/H_2O (60:40) flow rate 1 ml/ min. All chemicals used for extraction and purification were of analytical grade.

Cytotoxic assays.

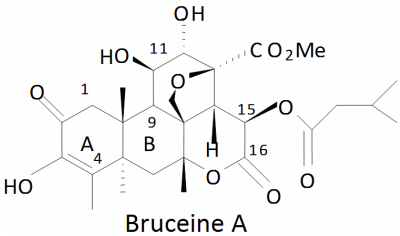
The cytotoxic activity (Mosmann, 1983) was tested by assessing their inhibition of growth of cells derived from human cancer. The cell lines tested was HeLa. Cells are adapted for growth in RPMI-1640 media supplemented with 10% bovine serum and 60 mg/mL amikacin and maintained 150 cm2 flasks at 37°C and 5% CO_2. Prior to assay for cytotoxic activity, cells are trypsinized, counted and suspended in media to a concentration of 250.000 cells/mL. An aliquot of 100 µL containing 25.000 cells was added to each well of a 96-well tissue culture treated microtiter plate. Media was added to adjust the volume. The cells are allowed to attach to the well for 24 hours in the incubator. After adherence, a 100 µL aliquot of sample compounds diluted in media was added to the medium in the wells. Samples are initially dissolved in DMSO and diluted in media such that the final concentrations in the wells are 383;191; 96; 47; 9.5; 2.5 µM. The final DMSO concentration was 0.5%. Cells are incubated with the samples for 24 hours. At 24 hours, the medium was removed from the wells and 100 µL of MTT (5mg/mL in RPMI) was added to each well. This dye was taken up only by viable cells. Following incubation for 4 hours, the dye was discarded and the cells are washed with physiological saline to remove extracellular dye. Isopropanol is added to each well to solubilize the cells and the dye. The absorbance at 595 nm was read and corrected for absorbance Elisa Reader.

Data Analysis. Percent (%) Growth calculated cell growth in treated cell by comparison to growth with no drugs and DMSO only using the formula: %Viable cells=[(cells with compound-media/cells in solvent-media)100]. IC_{50} value of sample for inhibiting 50 % of growing cells tested. IC_{50} value was determined by linier regression. The value of % death cell for both the treated and control were statistically analysed by Independent T-test at significant level $P<0.05$.

Result and Discussion

From this study we had the spectroscopic data of bruceine, the isolated compound of *B.javanica* from Indonesia. We then, to confirm these data and crosscheck with data published of bruceantine and brusatol analogues.

The structural identity of bruceine (Figure 1) from *B. javanica* was established by spectroscopic analysis. As a result from this study the CNMR of bruceine in ^{13}C NMR 75 MHz, Pyr (d6), at δ 192.7 4 (C-2), 167.94 (C-16), and 167.94 (C-1') were ketone carbon cyclic specific for brusatol analogues (Kim, 2004; Hitotsuyanagi, 2006). Chemical shift at 170.98 (C-21) specific for metoxy carbonyl but metoxy alone has at δ 52.05 (CH3O-22). Some carbon quartener at 127.96 (C-4), 122.0 (C-20), 83.46 (C-7), 82.42 (C-13). Any Shifted at 145.71 (C-3), 75.35(C-12), 72.79 (C-11) belonging to carbon attached with hydroxyl. Moderate to upfield chemical shifted from δ 67.32(C-15), 49.75 (C-1), 45.85(C-14), 42.96 (C2'), 42.07 (C-10), 41.90 (C-5), 40.98 (C-8), 30.16 (C-9), 29.19 (C-6), and 25.58 (C-3') were methins and methenes groups. Meanwhile methyls group had at lower δ 22.16 (C-4'), 22.06 (C-5')15.36 (C-19) and 13.08 (C-18).



Bruceine A

Figure 1. Structural chemisry of bruceine A (C_{26} H_{34} O_{11}).

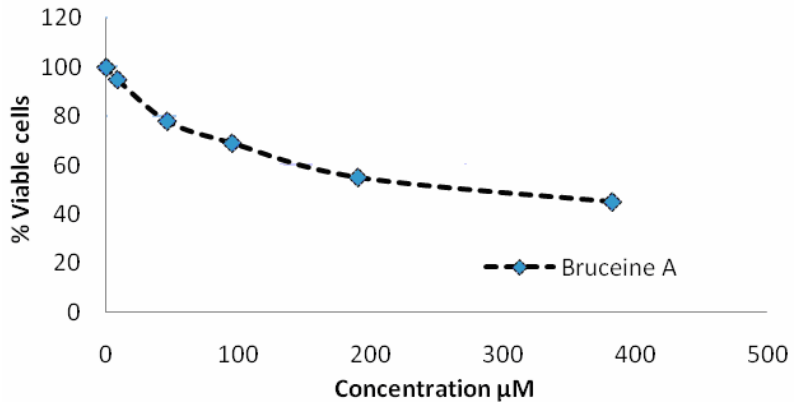


Figure 2. Profile cytotoxic effect of bruceine against HeLa cells at 24 hours.

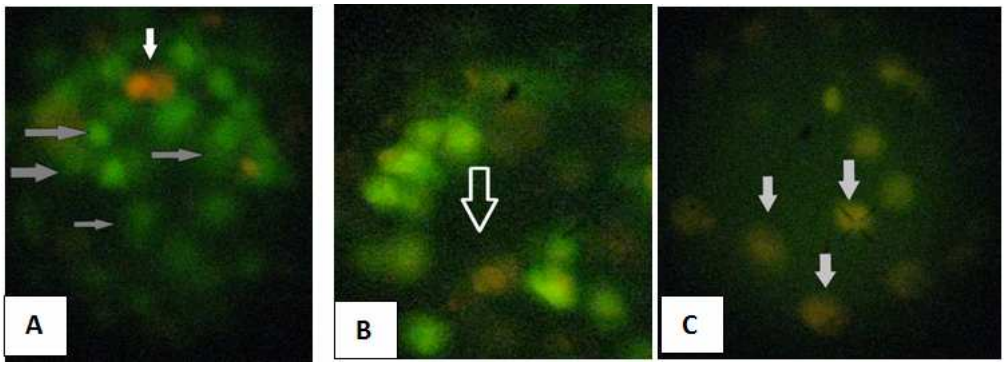


Figure 3. Effect of bruceine and doxorubicine induced apoptotic on HeLa cells staining by *etidium bromide-acrydine orange* (EB-AO).
A .Bruceine 191 µM. B. Bruceine 383 µM. C. Doxorubicine 96 µM.
Arrows ⬇ on photo possible apoptotic cells orange, arrows ➪ on cells normal.

The ^{13}C-NMR spectra showed that compound had 26 Carbons. The carbon confirming by Dept 30°;45° ;90° and 135°. It had four carbon methene (-CH_2) by DEPT determine in 135°. Two methyls showed in C-4 and C-10. Although both are methyls but differ in chemical shift belonging to bruceantine, brusatol and bruceine (Kim, 2004). The chemical shifts of Me-4 and Me-10 that we had in bruceine were (δ 1,93 and 1,72 respectively). The signal showed down field in Me-4 comparing to Me-10 confirmed that methyl-4 attached to carbon double bond. Therefore some reason that the chemical upfield shifted of Me-4 confirmed the esterification of C-3 or lost of double bond has occurred. Bisides methyls C-4 and C-10 of bruceine also had two methyls at ester side chain C-15. That two methyls at side chain ester C-15 bruceine appeared in upfield shifted compared to brusatol. In this study the HNMR spectra of bruceine A in ^{1}H NMR 300 MHz, Pyr (d5) and DMSO (d6) gave: δ2.46 (1H, d, *J*=16.9 Hz, Ha-1), 3.26 (1H, d, *J*=15.9Hz, Hb-1), 2.57 (1H, d, *J*=5.2 Hz, H-5), 2.18 (1H, ddd, *J*=14.1, 2.8, 2.6Hz, Ha-6), 1.74 (1H, ddd, *J*=16.1,6.9, 6.3Hz, Hb-6), 4.90 (1H, s, H-7) (dmso), 2.97 (1H, brd, *J*=7.9, H-9), 4.75 (1H, m, H-11), 4.07(1H, s, H-12) (dmso), 3.28 (1H,s,H-14), 6.51 (1H, brs, H-15), 1.61(3H, d, *J*=1.8, H-17) 1.19 (dmso), 1.93 (3H, d, *J*=1.8 Hz, H-18) 1.71,d, *J*=1.4Hz, (dmso), 3.79 (1H, d, *J*=7.5 Hz, Hb-20), 4.73 (1H, d, *J*=4.8, Ha-20), 3.66 (dmso) (3H, s, CH3O-21) 3.85 cdcl3, 0,88 (3H,d *J*=6.5Hz, H-4'), 0,90 (3H, d, *J*=6.9Hz, H- 5'), 3-OH, s, 7.82 (dmso), 11-OH, d, 5.98 (dmso),12-OH, brs, 5.56 (dmso).

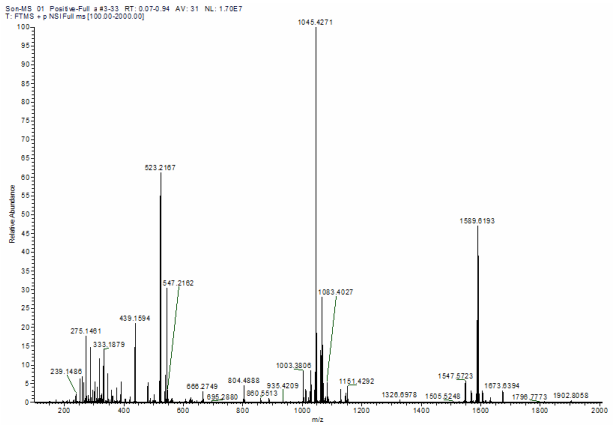


Figure 4. HR-ESIMS of bruceine (MW 522.135) in positive mode

The ^{1}H-4' and ^{1}H-5' protons showed signals at δ 0,98 and δ 0,95 doublets confirmed that those methyls were cymetric attached to carbon methin (C3'-H-) (Harigaya, 1989). In the ^{1}H-NMR spectra of bruceine Me-4' and Me-5' doublets signal appeared is lower compared to signals value of brusatol and bruceantin ($\delta_{H4'}$ at 1.93 and $\delta_{H5'}$ at 2.19). This fact the upfield shifted of Me-4' and Me-5' above confirmed that signals were methyls belong to bruceine. Protons analysis ^{1}H-NMR of hydroxyls at C-3, C-11 and C-12 more clearly showed in dmso (d6) solvent which δ 3-OH ,7.82 (dmso), 11-OH, d, 5.98 (dmso),12-OH, brs, 5.56 (dmso). Meanwhile the ^{1}H NMR proton of O-CH3 showed in sharp and high peak at δ 3,68-3,79 ppm at several solvent analysis. Some of quassinoid can be in forms of aglycone or glycone formation attached to ester side chain of hydroxyl at C-3 or C-2 to give glycoside. Its ^{13}C-NMR can be at δ (103.6, 78.9, 78.0, 74.5, 71.3, 62.3) ppm and the ^{1}H-NMR at δ (4.99, 4.01, 4.21, 4.23, 3.391, 4.53) ppm such a hexose unit (Kim, 2004).

Based on Analytical HPLC bruceine from with serial concentration treatment (150;100;50;25 ppm) showed retention time, t_R was 4.617 minutes (Met/H_20) 60:40. The flow rate of eluent was 1 mL/min using Lichrosorp RP-18 column. So far there is no data published of analytical HPLC of bruceine had found.

Bruceine as quassinoid was confirmed by Liberman-Burchard reaction test in the TLC plate to give pink colour of spot. The UV spectrum (Figure not shown) were determined on UV Milton Roy spectrophotometer 3000 showed the absorption maxima at λ_{max} 204 and 278 nm refers to *a,β* keto unsaturated group in quassinoid likes brusatol as well as brucein A (Silverstein and Webster, 1998). The Infra red spectral data of bruceine from *B. javanica* showed spectrum IR υ_{max} (KBr) cm^{-1} at : 3433, 2960, 1737, 1643, 1284, 1147, 1066, 1022, cm-^{1}). Infra Red (film) ν_{max} 3443 cm^{-1} (came from carbocyclic OH), 2960 (streching C-H), 1737 cm^{-1} (αβ unsaturaed C=O), 1643 cm^{-1} (C=C),1284 -1147 cm^{-1} vibration C-O), 1066 cm^1 - 1022 cm^{-1} (O-CH3 Metoxy). The crystal of bruceine is running into the melting point test by using microtube, melting point were recorded on a Scientific melting point apparatus Electrothermal 9100 array. It has 261-263 ^{o}C. The value was correspond to brucein A, with 267-270 ^{o}C. without no corrections. Analysis ESI-MS showed that molecular ion peaks at m/z ; 523,03$[M+H]^+$; 540,04. $[M+H_2O]^+$ were deduced from MW of bruceine A 522,1203 and might be attached with water to have m/z 540.04 $(M+H_2O)^+$.

These chemical shift assignments were obtained for bruceine from ^{1}H-NMR and ^{13}C-NMR data spectrum corresponding to the molecular formula (C_{26} H_{34} O_{11}). Additionally the obtained crystal was to analysis to know how far that activity of that compound as anticancer. As the purpose of this paper is to present the full account of the structural elucidation of brucein-A as well as anticancer activity.

Due to cell cancer activity the process of neoplastic cell can be depicted as a dysfunctional balance between control of cell proliferation, apoptosis and terminal differentiation. In normal cells activation of specific of pathways leads to cellular differentiation which typically is accompanied by cell growth arrest followed by apoptosis. The cell test had been chosen for HeLa cell line because of the high incidence of cancer cervix in the world. The effect of anticancer bruceine on the growth of Hela cell line were tested under *in vitro* conditions using serial concentration starting from 383 to 2.5 μM. After 24 hours at incubator, MTT was added. This dye was taken up only by viable cells. In a high concentration looked some of the death cell as cytotoxic effect of bruceine compare to the lowest concentration . The cells survival have a long live and more colored with MTT compared to death cells. Therefore reading the value of absorbance at 595 nm Elisa Reader of viable cells was higher then death cells. Due to death cell this analysis supported that cell mitochondrial membrane potential depolarization pathway was induced by brucein A (Lau *et al*., 2005). Additionally, any morphological change of cell tested in serial treatment concentration from low to high. At 383 μM doses of bruceine showed that cell treated toward morphological change became round when compare to control and some of those were disruption. The cytotoxic effect of brucein on cell HeLa and death cell has been occured. Death cell induction towards can be activated via Apo1-/Fas receptor, caspace 8; 9 and caspace 3. Study of Lau *et al.,* (2005) extract of *B. javanica*-induced cancer cell death proceed through a mitochondrial dependent pathway associated with caspace 3 activation. Previously study revealed that death cell tested can be via apoptosis, p53 and down regulated *c-myc* that induced by brusatol (Greenwod *et al*., 2002).

The IC_{50} value was calculated to be 191.5 μM for brucein A at 24 hours. Seems to be sensitive for Hela Cells line and can be followed in to 48 hours observation looking for advanced activity. Although in several preliminary study of bruceantine and brusatol had been known that IC_{50} value was ranged 4-25 ng/mL (Hitotsuyanagi *et al*., 2006, Greenwood *et al*., 2002). The trend line IC_{50} value bruceine can be seen at Figure 2.The cytotoxic activity was compared with the effect of doxorubicine of IC_{50} value 5.388 μM and its was found that cell was sensitive both to bruceine and doxorubicine. From our data the isolated active principle bruceine offered a high potent to inhibition of the *Hela* cells compared to cells T47D (data not showed).

Apoptotic assay also was done in this study. After 24 h incubation cells were stained by *etidium bromide-acrydine orange* (EB-AO). Control cells with no drugs showed in green florescent since some of cells treated showed in orange florescent. Based on apoptotic assay of bruceine and doxorubicine have a cytotoxic activity that induced by apoptotic (Figure 3).

From MS data ion peak at m/z 1045,4271 is 523 unit too high then 522, it may came from dimeric compound [M2+]. We suggest that formula m/z 1045,4271 was the two molecule parent together to form and the adduct ions $[2M+H]^+$. In mass spectra m/z 523 deduced molecule brucein A corresponds to the data.

The spectra on Figure 4 showed $(M+H)^+$ molecular weight 523,135 g/mol that are 1 mass unit too high from 522,125 g/mol. This due to one proton adduct on the molecular of bruceine $(M+)^+$.

Conclusion

In conclusion that due to spectral data FT-IR, ^{1}H NMR ,^{13}C NMR, HPLC and LC-MS showed that quassinoid bruceine A was existed in *B.javanica.* Anticancer activity assays toward Hela cells line showed the IC_{50} value of bruceine was 191,5 μM comparable to doxorubicine.

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